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Development of a Polymerase Chain Reaction Test for Specific Identification of the Urinary Tract Pathogen *Aerococcus urinae*

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A polymerase chain reaction test was developed for identification of the gram-positive urinary tract pathogen *Aerococcus urinae*. Oligonucleotide primers were based on highly specific sequences within the small-subunit rRNA gene. A confirmatory test based on hybridization of the amplified products to a highly specific internal probe was also developed.

Aerococci occur in air, dust, vegetation, hospital environments, and meat-curing brines. They are responsible for a fatal disease of lobsters, and although initially considered saprophytic to humans, they have also been recovered from patients with a number of clinical conditions, including endocarditis (5, 9), bacteremia (8), meningitis (7), osteomyelitis (11), and septic arthritis (10). Despite the diversity of habitats, aerococcal isolates show very similar phenotypic features. Although some genomic heterogeneity has been reported (2, 12), aerococci have been classified in a single species, *Aerococcus viridans*, for nearly 4 decades.

During a long-time survey, Christensen et al. (3, 4) recovered, at a low but constant frequency, some *Aerococcus*-like organisms from patients with urinary tract infections. Most of the patients were elderly and suffered from other systemic and localized conditions. Although sometimes present in mixed cultures, these *Aerococcus*-like organisms were considered opportunistic pathogens, owing to their recurrence and the presence of typical symptoms of urinary tract infections, which ceased following antibiotic treatment targeted at the *Aerococcus*-like organisms. These isolates had many characteristics in common with the genus *Aerococcus* but differed from *A. viridans* in several phenotypic traits. Subsequent small-subunit (16S) rRNA sequencing studies revealed that these clinical isolates constitute a separate line of descent within the genus *Aerococcus* for which a new species, *A. urinae*, was proposed (1).

It is recognized that identification of aerococci is complex and time-consuming. Not only is there considerable phenotypic overlap with pediococci and other lactic bacteria, with misidentification occurring most frequently with streptococci, but aerococci are characterized by the lack of features present in other taxa. Identification of aerococci from clinical sources is usually based upon a battery of phenotypic traits, including morphology; the catalase test; growth in pH 9.6, in 6.5 and 10% salt, and in 40% bile; and other biochemical reactions. Cellular fatty acid composition, vancomycin susceptibility, and the pyrrolidoylarylamidase test have also been proposed (2). In this study, we developed a polymerase chain reaction (PCR) probe test based on 16S rRNA for specific identification of the newly described urinary tract pathogen *A. urinae*.

Details of the *A. urinae* and reference strains used are given in Table 1. We grew aerococci, enterococci, lactococci, and streptococci in yeast glucose phosphate broth, staphylococci in nutrient broth (Oxoid Ltd.), and *Gemella haemolysans* in Todd-Hewitt broth (Oxoid Ltd.). Lactobacilli, leuconostocs, and pediococci were cultivated in MRS broth (Oxoid Ltd.). DNA for PCR amplification was extracted from late-exponential-phase cultures by a modification of the method of Lawson et al. (6). The DNA was quantified spectrophotometrically (A_{260}), and purity was checked by 0.8% agarose gel electrophoresis. PCR was also performed on crude cell lysates prepared by suspending a single colony in 100 μl of sterile distilled water, vortexing it for 2 min with glass beads, and boiling it for 5 min.

Three specific oligonucleotides were designed from the 16S rRNA sequence of *A. urinae* for use as PCR primers and/or as probes in hybridization experiments: Au1 (5′-AGGAAGGTCACCGCATGGTGA), Au2 (5′-CATGGTGA CCTTTGGAAAGACG), and Au12 (5′-TTCGCTTGCTTTC ACAAGGTC). Two universal primers, ARI (5′-GAGAGTT TGATCCTGGCTCAGGA, positions 8 to 30 [*Escherichia coli* numbering system]) and EE (5′-TTCGAATTAAACCA CATGC, positions 966 to 948), derived from highly conserved regions of the 16S rRNA, were used as PCR-positive controls. A third universal primer, pH (5′-AAGGAGGTG ATCCAGCCGCA, positions 1540 to 1521), was employed in combination with ARI to generate rDNA PCR fragments for use in slot blot hybridizations. PCR reaction mixtures (50 μl) contained 5 μl of 10× buffer (50 mM KCl, 10 mM Tris-HCl, 15 mM $MgCl_2$, 10% [vol/vol] glycerol), 5 μl of deoxyribonucleotide mixture (1.25 mM each), 1 μl of each primer (0.2 mM), 1 μl of DNA (final concentration, 10 ng/50 μl), and sterile water to volume. The mixture was denatured at 94°C for 3 min before addition of 1 μl (0.5 U) of *Taq* polymerase (Amersham International, Amersham, United Kingdom). The PCR temperature profile consisted of 25 cycles of 2 min of denaturation at 92°C, 1 min of annealing at 58°C, and 1.5 min of primer elongation at 72°C; in the last cycle, the primer extension was continued for 5 min.

Oligonucleotide Au2 was used as a confirmatory probe in Southern blot hybridizations with products generated with primer combination Au1-Au12. After electrophoresis, the

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TABLE 1. Aerococci and reference strains included in this study

Species	Strain[a]	Species	Strain[a]
Aerococcus urinae	NCFB 2893^T	*L. lactis*	NCDO 604^T
A. urinae	CCUG 28940	*L. raffinolactis*	NCDO 617^T
A. urinae	CCUG 29552	*L. piscium*	NCFB 2778^T
A. urinae	CCUG 29553	*L. plantarum*	NCDO 1869^T
A. urinae	CCUG 29554	*Leuconostoc citreum*	NCDO 1837^T
A. urinae	CCUG 29558	*L. lactis*	NCDO 533^T
A. urinae	CCUG 29591	*L. mesenteroides*	NCDO 523^T
A. urinae	CCUG 29563	*L. paramesenteroides*	NCDO 803^T
A. urinae	CCUG 29569	*Enterococcus avium*	NCDO 2369^T
A. urinae	B3[b]	*E. casseliflavus*	NCDO 2372^T
A. urinae	I3[b]	*E. faecalis*	NCDO 1118
A. viridans	NCDO 1225^T	*E. faecium*	NCDO 942^T
A. viridans	ATCC 10400	*E. gallinarum*	NCDO 1229
A. viridans	CCM 2439	*E. hirae*	NCDO 1258^T
A. viridans	NCDO 1806	*E. saccharolyticus*	NCDO 2612
A. viridans	NCDO 1807	*Streptococcus agalactiae*	NCDO 1348^T
A. viridans	NCDO 1809	*S. alactolyticus*	NCDO 1091^T
A. viridans	NCDO 1840	*S. anginosus*	NCDO 2496^T
A. viridans	NCDO 1841	*S. equinus*	NCDO 2615
A. viridans	NCDO 1843	*S. mutans*	NCDO 2062^T
A. viridans	NCDO 1224	*S. oralis*	NCDO 2680^T
A. viridans	CCUG 15548	*S. pyogenes*	NCDO 2381^T
A. viridans	CCUG 22203	*S. salivarius*	NCDO 1779^T
A. viridans	1500-81[c]	*S. sanguis*	NCDO 2106
A. viridans	153-87[c]	*S. suis*	NCDO 2644
A. viridans	356-90[c]	*S. uberis*	NCDO 2038^T
Tetragenococcus halophilus	NCDO 1635^T	*Lactobacillus casei*	NCDO 161^T
Gemella haemolysans	NCTC 10243^T	*L. confusus*	NCDO 1586^T
Pediococcus damnosus	NCDO 1832^T	*L. plantarum*	NCDO 1752^T
P. dextrinicus	NCDO 1247^T	*L. rhamnosus*	NCDO 243^T
P. pentosaceus	NCDO 990^T	*Staphylococcus aureus*	NCDO 949^T
P. urina-equi	NCDO 1636^T	*S. epidermidis*	NCTC 11047^T
Lactococcus garvieae	NCDO 2155^T		

[a] Abbreviations: NCDO, National Collection of Dairy Organisms; NCFB, National Collection of Food Bacteria; NCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection; CCM, Czechoslovak Collection of Microorganisms; CCUG, Culture Collection of the University of Gothenburg.
[b] Received from J. J. Christensen, Copenhagen, Denmark.
[c] Received from R. R. Facklam, Centers for Disease Control, Atlanta, Ga.

gel was rinsed in distilled water and then placed in denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 30 min and in neutralization buffer (1.5 M NaCl, 0.5 M Tris-HCl [pH 7.2], 0.001 M EDTA) for a further 30 min, both with agitation. DNA fragments were transferred to a nylon membrane (Hybond N^+; Amersham International) by capillary blotting for 2 h. The membrane was alkali fixed (0.4 M NaOH) for 20 min, rinsed in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and stored at 4°C in Saran Wrap (Genetic Research Instrumentation, Dunmow, United Kingdom). The probe was labelled with fluorescein by using the ECL 3′-oligotailing system (Amersham International) and hybridized to the target DNA at 42°C for 2 h in the recommended hybridization buffer. Stringency was controlled by washing the membrane twice in 5× SSC–0.1% sodium dodecyl sulfate (SDS) (5 min each time) at room temperature and in 1× SSC–0.1% SDS (15 min) at 50°C.

All three of the oligonucleotides used in PCRs were also tested as probes in slot blot hybridizations. Amplified rDNA products used in these hybridizations were generated with 16S rRNA universal primers ARI and pH. The PCR products were denatured by boiling for 3 min, cooled in ice, and applied to a Hybond N^+ membrane (Amersham International) with the Bio-Rad slot blot apparatus (Bio-Rad, Hemel Hempstead, United Kingdom). Probe labelling, hybridization, washing, and detection were performed as described above, by using the ECL 3′-oligotailing system. Membranes were stripped, prior to reprobing with universal probe EE, by boiling in 0.5% SDS for 8 min and washing in 2× SSC for 5 min.

On the basis of comparative analysis of available 16S rRNA sequences, three diagnostic oligonucleotides were designed: Au1 and Au2 were derived from variable region V2, and Au12 was derived from variable region V8. The oligonucleotides were used as primers for PCR in two combinations: Au1-Au12 and Au2-Au12. PCR was performed with purified DNA or crude cell extracts. Both primer combinations amplified the *A. urinae* DNAs successfully, generating PCR fragments of ca. 1,000 bp. The specificity of the two primer pairs was tested in studies with DNAs from aerococci, enterococci, pediococci, streptococci, and some other low-G+C-content gram-positive bacteria (Table 1). Only DNAs from *A. urinae* strains were amplified, generating PCR products of the expected sizes, while DNAs from *A. viridans* and all of the other reference species examined gave negative reactions. Figure 1a shows the results obtained with primer combination Au1-Au12. Identical results were produced with the other pair (data not shown). Positive controls run with two universal 16S rRNA primers indicated that the DNAs were of good quality and ensured that no inhibition of PCR had occurred (data not shown). Oligonucleotide Au2 was also utilized in a confirmatory hybridization test for PCR primer pair Au1-Au12. Southern blot hybridization with Au2 as an internal probe is

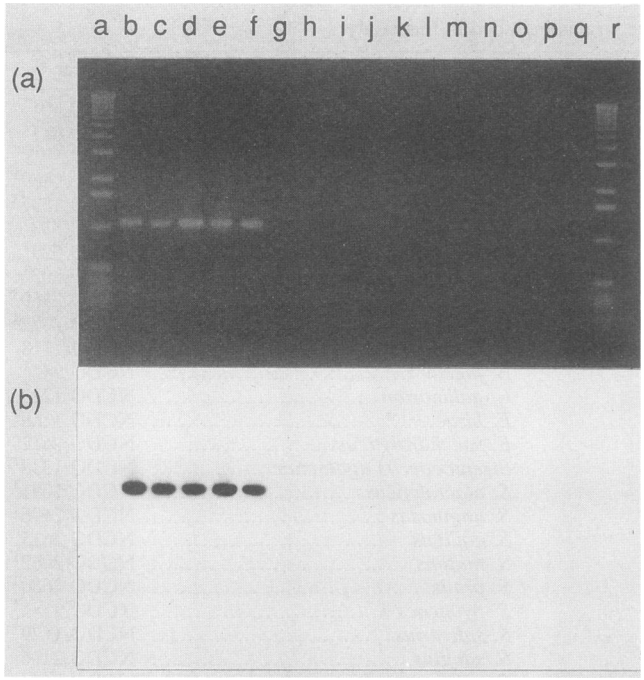

FIG. 1. (a) Agarose gel electrophoresis showing amplification of DNAs from representative reference strains with specific primer pair Au1-Au12. Lanes: a, 1-kb λ DNA ladder; b, *A. urinae* NCFB 2893; c, *A. urinae* CCUG 28940; d, *A. urinae* CCUG 29553; e, *A. urinae* CCUG 29563; f, *A. urinae* B3; g, *A. viridans* NCDO 1225; h, *A. viridans* ATCC 10400; i, *A. viridans* CCM 2439; j, *A. viridans* CCUG 22203; k, *A. viridans* 1500-81; l, *Pediococcus urina-equi* NCDO 1636; m, *Tetragenococcus halophilus* NCDO 1635; n, *Streptococcus sanguis* NCDO 2106; o, *S. pyogenes* NCDO 2381; p, *S. mutans* NCDO 2062; q, negative control; r, 1-kb λ DNA ladder. (b) Autoradiograph of the Southern blot after hybridization to oligonucleotide probe Au2.

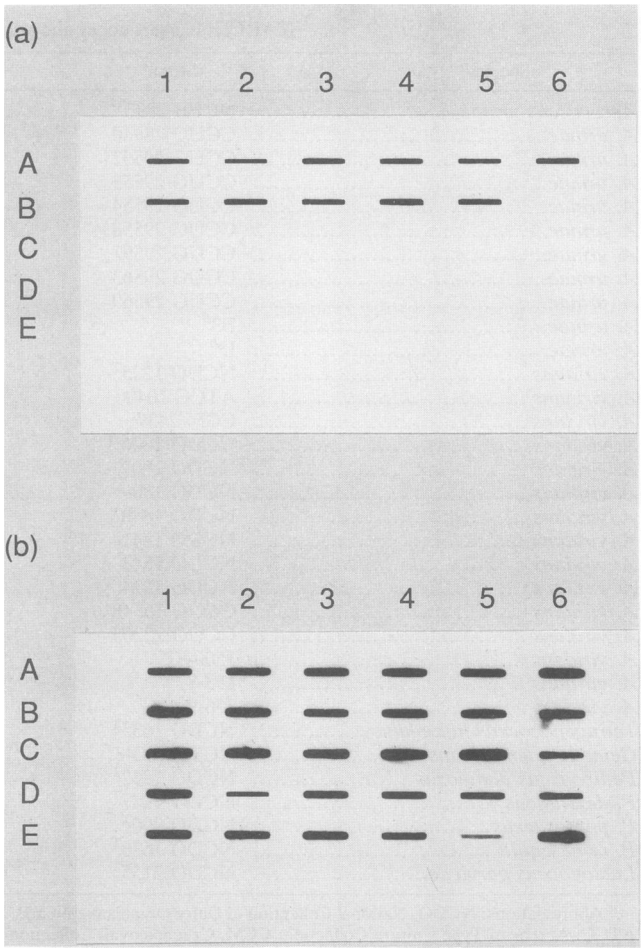

FIG. 2. (a) Autoradiograph of slot blot hybridizations to amplified 16S rDNA fragments using oligonucleotide Au12. (b) Autoradiograph of the same slot blot hybridized to universal 16S rRNA oligonucleotide EE. Slots: A1, *A. urinae* NCFB 2893; A2, *A. urinae* CCUG 28940; A3, *A. urinae* CCUG 29552; A4, *A. urinae* CCUG 29553; A5, *A. urinae* CCUG 29554; A6, *A. urinae* CCUG 29558; B1, *A. urinae* CCUG 29561; B2, *A. urinae* CCUG 29563; B3, *A. urinae* CCUG 29569; B4, *A. urinae* B3; B5, *A. urinae* I3; B6, *A. viridans* NCDO 1225; C1, *A. viridans* ATCC 10400; C2, *A. viridans* CCM 2439; C3, *A. viridans* NCDO 1806; C4, *A. viridans* NCDO 1840; C5, *A. viridans* NCDO 1224; C6, *A. viridans* CCUG 15548; D1, *A. viridans* 153-87; D2, *A. viridans* 1500-81; D3, *P. urina-equi* NCDO 1636; D4, *T. halophilus* NCDO 1635; D5, *G. haemolysans* NCTC 10243; D6, *P. pentosaceus* NCDO 990; E1, *Leuconostoc lactis* NCDO 533; E2, *Enterococcus faecalis* NCDO 1118; E3, *Streptococcus salivarius* NCDO 1779; E4, *S. pyogenes* NCDO 2381; E5, *S. sanguis* NCDO 2106; E6, *S. mutans* NCDO 2062.

shown in Fig. 1b. PCR products of *A. urinae* strains gave intense positive reactions with this probe. In contrast, PCR fragments generated from amplification of reference strain DNAs by using universal 16S rRNA primers failed to hybridize with probe Au2 (data not shown).

Although no cross-reactions were observed in the PCR tests, the specificity of the three primers-probes was confirmed by slot blot hybridizations. rDNA products generated with universal primers ARI and pH were immobilized on nylon membranes and hybridized with the 3′-end-labelled primers. The results of slot blot hybridization with probe Au12 are shown in Fig. 2a. All of the strains of *A. urinae* examined proved positive with this probe, whereas *A. viridans* and other reference species failed to hybridize. To eliminate the possibility of false-negative results, all blots were stripped and reprobed with universal 16S rRNA probe EE (Fig. 2b). Although not shown, identical results were obtained with probes Au1 and Au2, confirming their high specificity for *A. urinae*.

The recently described species *A. urinae* (1) is an opportunistic pathogen causing urinary tract infections in patients which, in the main, show predisposing conditions (3, 4). This species may also be responsible for other invasive infections with increased risks, such as endocarditis. In this study, we developed a simple molecularly based test for specific identification of *A. urinae*. The method is based upon species-specific PCR amplification of fragments of rDNA by utilizing characteristic sequences within the variable regions of the 16S rRNA gene. *A. urinae* constitutes a new addition to the catalase-negative, gram-positive group of organisms that diagnostic laboratories must be able to identify. The test described here does not require use of hybridization probes and should provide reference laboratories where PCR facilities are available with a rapid and highly specific means of identifying this new urinary tract pathogen.

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